Thesis for the Degree of Master of Fisheries Science

Fermentation of Nile tilapia (*Oreochromis niloticus*) wastes using *Lactobacillus plantarum* for the production of lactic acid and fertilizer

by

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KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

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젖산과 비료를 생산하기 위한 *Lactobacillus plantarum* 을 이용한 나일 틸라피아(*Oreochromis niloticus*) 폐기물의 발효

Advisor: Prof. Kim Joong Kyun

by

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Abstract

The waste (heads, bones, skin, scales, fins and guts) of Tilapia (*Oreochromis niloticus*) was hydrolyzed by concentrated H_2SO_4 acid, and then the supernatant was obtained after centrifugation and filtration. The supernatant was divided to three samples. First sample, only the acid-hydrolyzed Nile tilapia fish wastes supernatant, second sample the acid-hydrolyzed Nile tilapia fish wastes supernatant including 1% glucose and the third sample which was the acid-hydrolyzed Nile tilapia fish wastes supernatant including 2% glucose. After that, the samples were inoculated with *Lactobacillus plantarum* bacteria and incubated at 30˚C. For the analysis, four parameters were tested every eight hours. These parameters were pH, lactic acid concentration, glucose concentration and the growth of bacteria. The results of the study indicated that the productivity of lactic acid in the first sample was not effective 5.5 g/l but those in second and third sample were 12.28g/L. and

16.1 g/L. respectively. In addition the results showed that the bacterial growth in the second sample was much higher than the other samples. For the germination test and fertilizing ability test, the pH of the fermented broth was equalized by adding 1M NaOH and diluted by distilled water to reduce the salinity to 10, 6 and 3‰. The best results were obtained from the diluted solutions at both 3 and 10‰ salinities as the GI% were 95 and 75 respectively. According to the results of the germination test, a hydroponic culture system was held using barley seeds to study the fertilizing ability of the final fermented broth. The diluted solution at 10‰ salinity was used against distilled water as a control. The results showed that the stem length of barley was 10.3 cm after 10 days culture on the diluted fermented broth, which was better than that (8.7 cm) of the control

.

1. Introduction

Lactic acid bacteria are being used in the production of numerous/ various fermented food products and they contribute to important qualities such as, taste, texture and shelf life (Carr et al. 2002). One of the most widespread Lactobacillus species which is used in food technology is *Lactobacillus plantarum*. It has a homofermentative metabolism, a high acid tolerance, and it's like most of the lactic acid bacteria needs a rich media containing compounds, such as peptides, vitamins and amino acids like arginine, leucine, isoleucine, tyrosine, valine and pantothenic acid.

Most of the laboratories use the MRS medium for the growth of the *Lactobacillus* bacteria. The MRS medium can not be used in commercial scale, because it will increase the cost of the production. So, in order to reduce the production cost the researchers started to test many materials for the growth of the bacteria.

Using of the fish wastes as a growth medium for bacteria did not take that big concern, but some studies tested the growth of microorganisms on

media containing fish materials and the results were encouraging (Dufosse et al. 2001; Poernomo and Buckle 2002; Vazquez et al. 2004; S.J. Horn et al. 2005).

Nile tilapia is a tropical species that prefers to live in shallow water. The lower and upper lethal temperatures for Nile tilapia are 11-12°C and 42°C, respectively, while the preferred temperature ranges from 31-36 °C. It is an omnivorous grazer that feeds on phytoplankton, aquatic plants, small invertebrates, benthic fauna, detritus and bacterial films associated with detritus.

Moreover, Nile tilapia has many advantages in comparison to other kinds of fish. It can adapt to harmful environmental conditions, low price, good taste, capability for intensive culture and high percentage of meat protein. For that reason, it is cultured in many countries all over the world especially in Egypt and China. As it was reported in the global aquaculture, Tilapia production, in 2003 was 1,272,012 tons. China came in the first place by 806,000 tons (63.3%) and Egypt was the second country by producing 200,000 tons (15.7%) (FAO Fishery And Aquaculture Statistics 2014). Also it is now popular for processing to produce fillet and frozen products.

In Egypt, Nile tilapia is the most common fish especially from aquaculture. In 2013, Egypt produced 1,454,401 tons of fish and the production of tilapia was 738,645 tons which is about 50% of the total production (GAFRD 2013). And as (Dale N.M. et al. 2004) mentioned during processing in Ecuador about 69.5% of tilapia body weight is waste tissue and only 30.5% is edible fillet. Also in Honduras, only 36% of body weight can be used as a fillet and 64% is waste (Ponce, L. E., and A. G. Gerant 2002). For Egypt, nearly about 490,000 tons of Tilapia fish are wastes and need treatment in order to become useful products and also to avoid environmental pollution.

Treating the wastes by fermentation using lactic acid bacteria is a good method because it is more efficient and easier than using yeast extract. Additionally, the produced lactic acid has an added value because it is used in many industrials.

Thus, the aims of this study are:

1- Environmental side through avoiding the pollution that causes from the fish wastage.

2- Economically side which is reflected in:

- The establishment of factories and units for fermentation and lactic acid production.
- The wastes residues after fermentation can be used as a cheap and high quality fertilizer.
- The production of lactic acid which has a lot of benefits like:

- Cosmetics & medicine industrials.
- Food & beverages industrials.
- Textile & leather industrials.
- Adhesives & solvents industrials.

3- Social side which is reflected in:

- Creating new investments.
- Reducing the percentage of unemployment.
- Create jobs opportunities and training in new branch.

2. Materials and Methods

2.1. Microorganism

I used *Lactobacillus Plantarum* bacteria, which are rod-shaped, gram-positive lactic acid bacteria. This kind of bacterium is commonly found in humans and other mammalian gastrointestinal tracts, saliva, and various food products. It can grow in temperatures between 15-45˚C, the optimum is 37˚C; It also needs pH levels as low as 3.2, but the optimum is 6.2 - 6.6.

L. plantarum is facultative heterofermentative that ferments sugars to produce lactic acid, ethanol or acetic acid, and carbon dioxide under certain conditions and selective substrates. Depending on the carbon source, these bacteria can switch from using heterofermentative to homofermentative ways of metabolism. This bacterium is acid and bile salt tolerant, which allows it to survive the passage through the gastrointestinal tract of humans. *L. plantarum* is the current interest of researchers and food scientists since it is considered a safe probiotic. Specifically, it can help limit the amount of pathogenic bacteria or diseases that can have a negative impact on humans.

In addition, recent research indicates that *L. plantarum* can be used as a vaccine.

For the storage of the bacteria, it was cultured in Petri dishes and incubated at 30˚C for 24 hours. Then it was sub-cultured in 5ml. glass tubes and incubated at 30˚C for 12 hours in a shaking incubator. After that, the glass tube sub-cultured to a 50 ml. flask in the shaking incubator at the same temperature for 6 hours. Finally, it was centrifuged for 20 min. and the seeds were transferred by pipette to small plastic tubes adding of 10% glycerol (1:1) and stored in deep freezer at -70˚C.

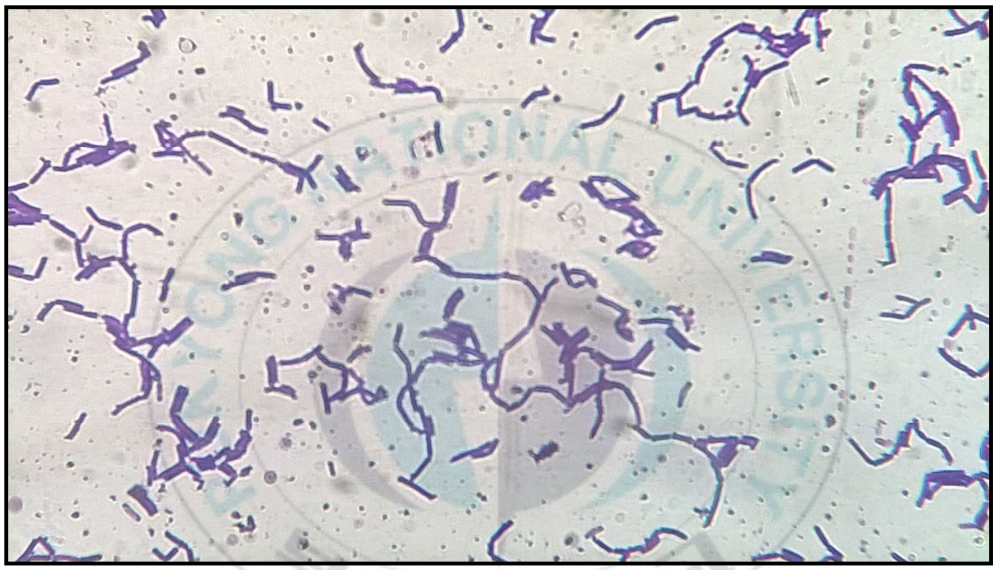

Fig. 1. *Lactobacillus plantarum* under the microscope.

2.2. Target Fish

The target fish in this study is Nile Tilapia *(Oreochromis niloticus)* which was obtained from PKNU aquaculture farm, because it is the most popular fish in Egypt and also it is rich in the amino acids, as it was measured in the fish meal during the study that was held by N. M. Dale on 2004.

Amino acid	Average (%)	Low (%)	High (%)
Lysine	3.37	3.01	3.59
Methionine	1.18	1.02	1.31
Cystine	0.36	0.31	0.41
Threonine	2.03	1.87	2.11
Tryptophan	0.39	0.36	0.43
Arginine	3.67	3.49	3.85
Valine	2.19	1.96	2.33
Isoleucine	1.72	1.56	1.86
Leucine	3.18	2.98	3.34
Histidine	1.07	0.95	1.15

Table 1. Composition of some amino acids in Tilapia fish meal.

2.3. Fish waste

Fish heads, bones, skin, scales, fins and guts were taken from a whole fish. The wastes were minced in a chopper with adding of 150 ml. distilled water. Half of the wastes were used directly and the other half were stored in freezer for future use.

2.4. Fermentation medium

In this study were used three fermentation experimental media and one control medium. The control medium was MRS (Difco™) which contains per litre 10 g Proteose Pepntone No.3; 10 g Beef Extract; 5 g Yeast Extract; 20 g Dextrose; 1 g Polysorbate 80; 2 g Ammonium Citrate; 5 g Sodium Acetate; 0.1 g Magnesium Sulfate; 0.05 Manganese Sulfate; 2 g Dipotassium Phosphate and pH 6.5. The experimental media were 3, (A) the supernatant of the acid hydrolyzed fish wastes without any additives, (B) the supernatant of the acid hydrolyzed fish wastes with addition of 1% glucose and sample (C) the supernatant of the acid hydrolyzed fish wastes with addition of 2% glucose.

Fig. 2. The experimental samples from the acid hydrolyzed fish waste.

2.5. Recovering microorganism

The stored microorganisms were recovered by tacking a scratch from the frozen seeds by a sterilised loop and cultured on MRS agar in Petri dish and incubated at 37˚C for 24 hours.

2.6. Culture conditions

The wastes needed to be hydrolyzed by acid first in order to extract the nutrient component in the supernatant, which will be used as a media for the *lactobacillus plantarum* bacteria for growth and the addition of alkaline to adjust the pH. Before adjusting the pH, glucose has been added due to the concentrations that were mentioned before.

Fermentation was performed in a 500 ml jars with a controlled temperature at 30˚C and pH 6.8 at the start of the experiment which was adjusted by addition of NaOH (concentration 1M). The fermentation was held in a shaking incubator with rotation speed of 150 rpm.

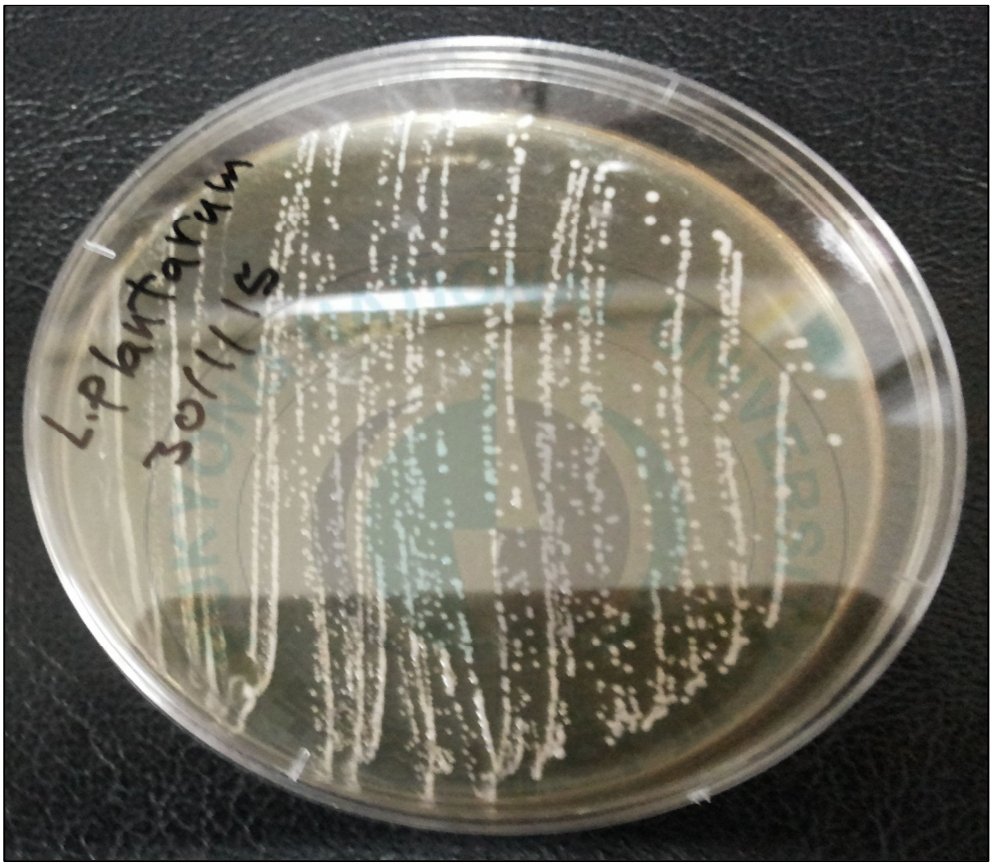

Fig. 3. Recovered *Lactobacillus plantarum* bacteria

2.7. Acid-hydrolysis

A 200 g. of minced wastes were put in a 1 L. glass jar and mixed with distilled water (1:2) and the pH was adjusted to 1 by adding concentrated H_2SO_4 acid (98%), then the sample was autoclaved at 121˚C for 15 min. After that, the sample was centrifuged for 15 min at speed 12000 rpm. Finally, the sample was filtered by a filter paper in another glass jar to get the supernatant, and adjust the pH to 6.5 by addition of NaOH (concentration 1M) then autoclaved again at 121˚C for 15 min.

The supernatant was used as a nutrient source for the production of lactic acid and the residue could be used as fertilizer after neutralization.

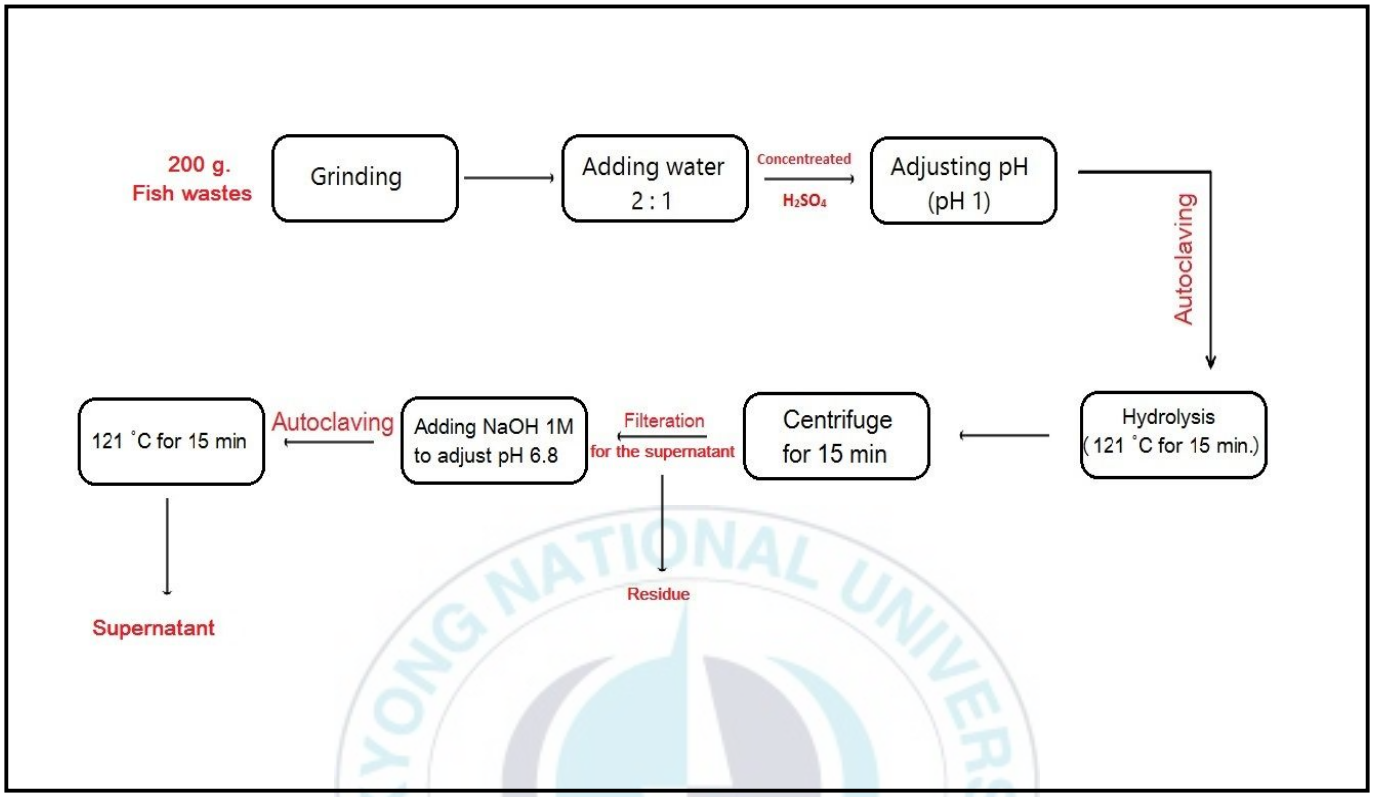

Fig. 4. Flow sheet of the acid-hydrolyzed Nile tilapia wastes supernatant preparation.

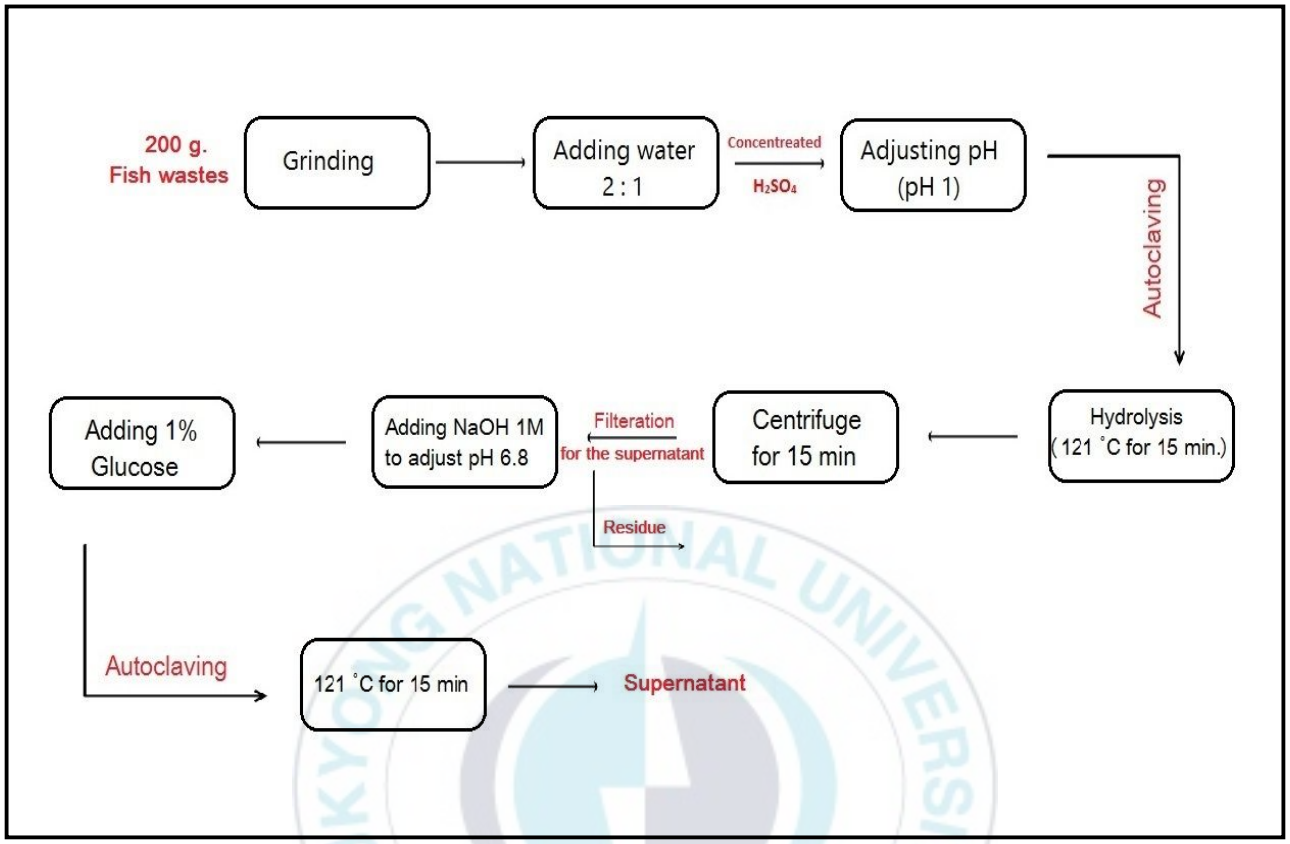

Fig. 5. Flow sheet of the acid-hydrolyzed Nile tilapia wastes supernatant including 1% glucose preparation.

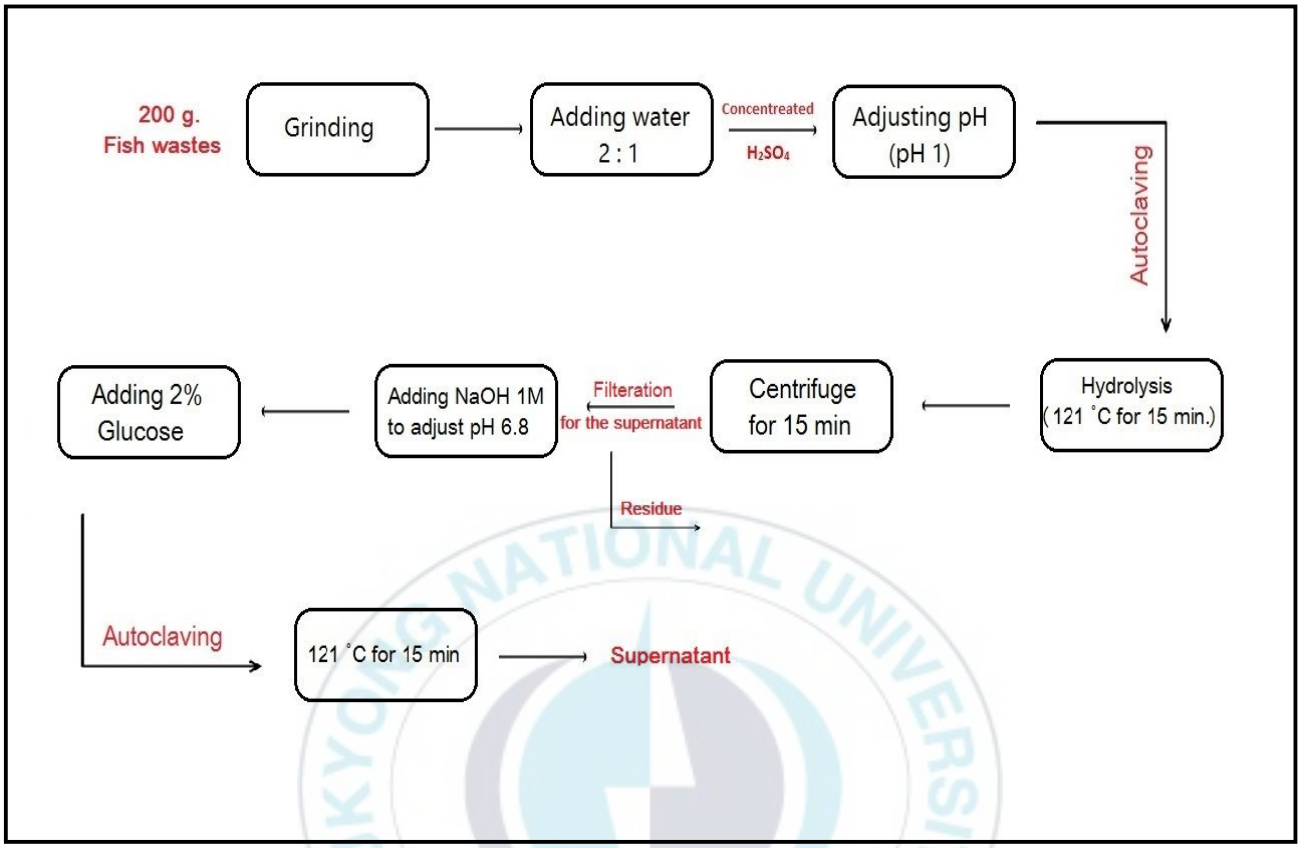

Fig. 6. Flow sheet of the acid-hydrolyzed Nile tilapia wastes supernatant including 2% glucose preparation.

2.8. Analysis

This study relied on four parameters which were measured every eight hours (three times per day). These parameters are concentration of lactic acid, which detected by titration with 0.1M NaOH solution and using of Phenolphthalein as indicator, pH which measured by pH meter, glucose concentration which detected by DNS method through adding 3ml of DNS solution to 3ml of sample. Heating for 5-15 min in 90 ˚C water bath untill the color turned to red or brown-red, then cool in a cold water till reach the room temperature. After that, adding of 1 ml Rochelle solution then measure by spectrophotometer at 575 nm wave length. Finally, the bacterial growth was determined by using Colony Forming Unit method (CFU).

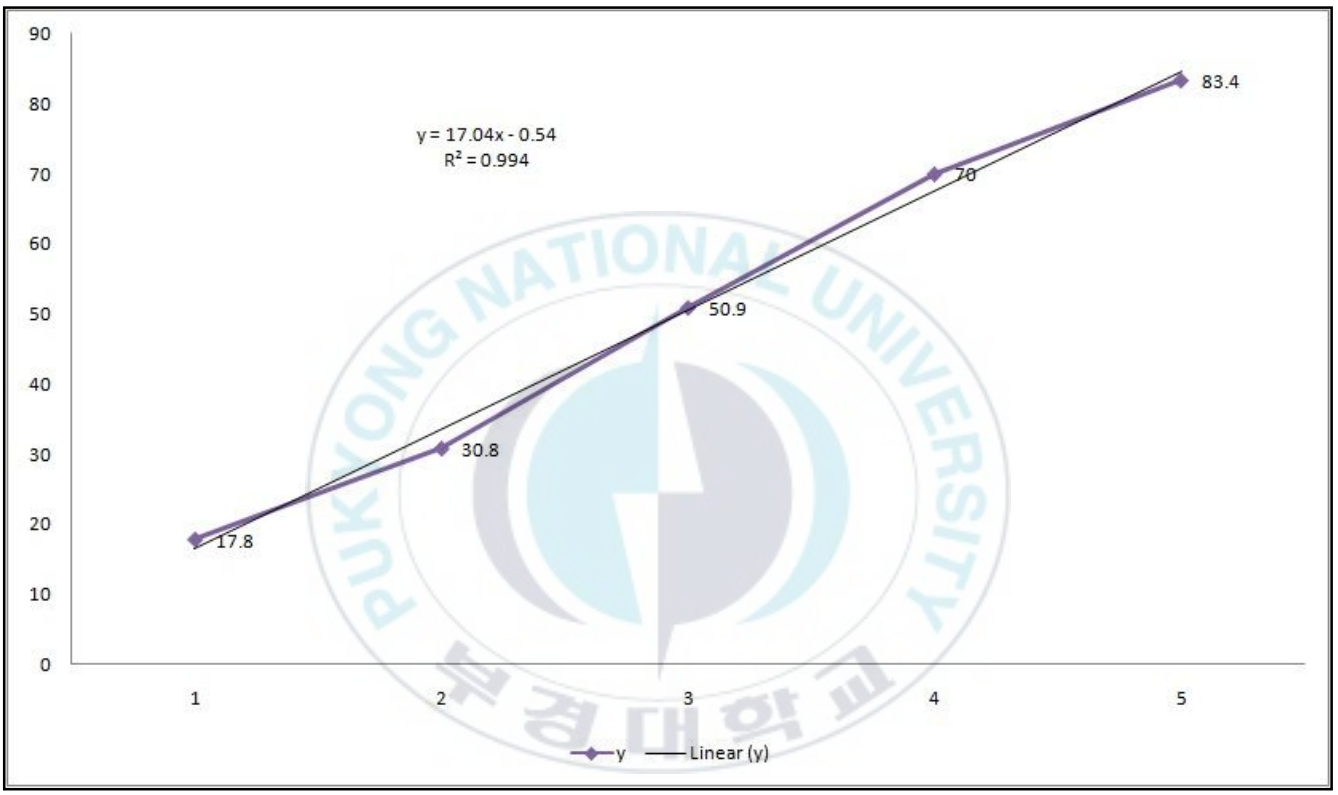

Fig. 7. Standard curve for calculating the concentration of lactic acid.

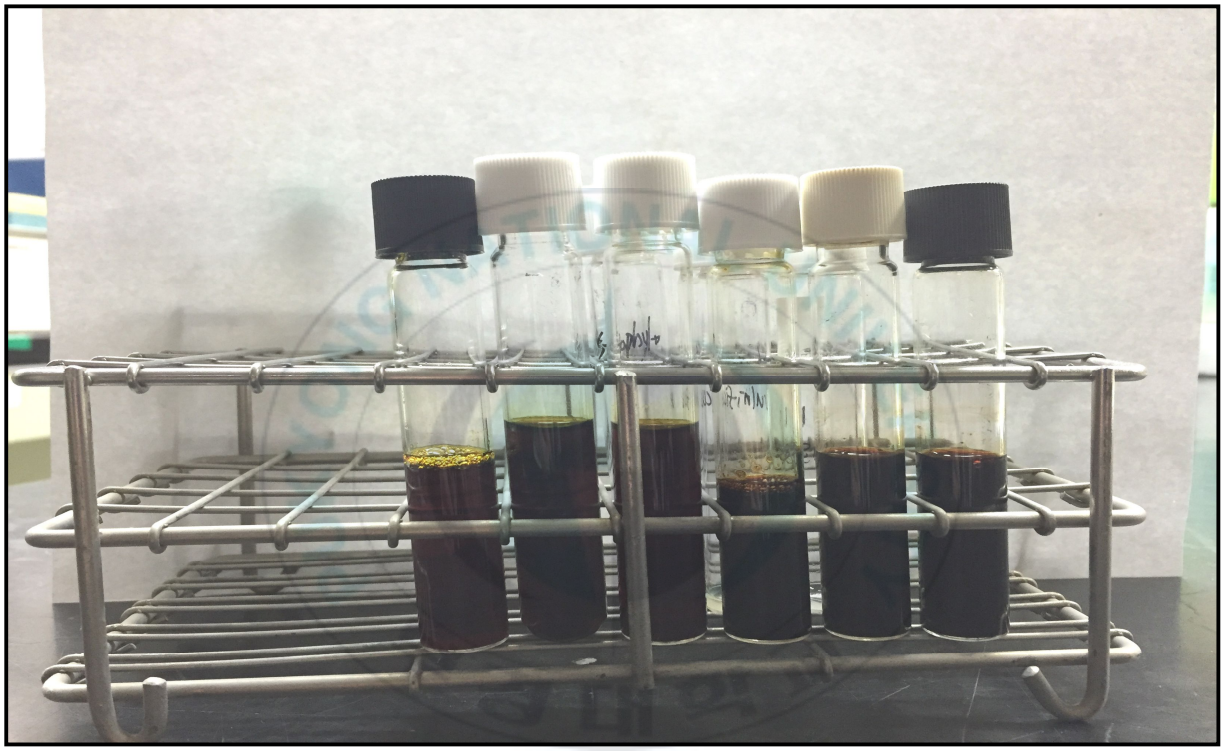

Fig. 8. DNS test for measuring the produced sugar.

2.9. Seed Germination test

After fermentation, the pH of the broth was equated by adding NaOH 1M to increase the pH level to 6.5-7 in order to become suitable for the plants growth. After the equation of the pH level, the salinity of the broth was increased to 50‰ which can not be used by any way for the growth of the plants. So, the broth was diluted by distilled water to decrease the salinity to 10‰, 6‰ and 3‰ respectively. In order to evaluate the phytotoxicity of the fermented Nile Tilapia fish waste, the previous dilution percentages were tested against the control sample which was held by using distilled water. Five milliliters of each sample were pipetted into a sterile Petri dish lined with Whatman #1 filter paper.

Ten cress (Lepidium sativum) seeds were evenly placed in each dish. The plates were incubated at 25˚C in the dark at 75% of humidity. Seed germination and root length in each plate were measured after 72 hours.

The percentages of relative seed germination (RSG), relative root growth (RRG) and germination index (GI) after expose to the sample were calculated as the following formula:

$$RSG\ (\%) = \frac{Number\ of\ seeds\ germinated\ in\ Nile\ Tilapia\ fish\ waste\ broth}{Number\ of\ seeds\ germinated\ in\ control} \times 100$$

$$RRG\ (\%) = \frac{Mean\ root\ length\ in\ Nile\ Tilapia\ fish\ waste\ broth}{Mean\ root\ length\ in\ control} \times 100$$

$$GI\ (\%) - \frac{RSG \times RRG}{100}$$

2.10. Hydroponic culture

To test the fertilizing ability of the fermented Nile Tilapia fish waste, a hydroponic culture system was applied to cultivate barley in a mini-hydroponic culture pot (5×12×8 cm^3) against control. Tests were carried out on the fermented Nile Tilapia fish waste at various dilutions according to salinity level. The hydroponic culture pot was composed of a plastic vessel and a plastic screen inside. In each pot twenty seeds of barley were put on the top of the plastic screen, and approximately 375-ml of the fermented Nile Tilapia fish waste diluted solution was filled underneath the plastic screen. The seeds were soaked all the time and roots grew through the pore of the plastic screen after seed germination. Before setting-up the hydroponic culture system, the seeds were incubated at 25˚C in the dark at

75% of humidity in order to germinate. After seed germination, the hydroponic culture system was applied and the temperature was controlled at 25˚C and the humidity was controlled at 75%. The fermented Nile Tilapia fish waste diluted solution was refreshed every 2-3 days as needed. The growth of plants was observed periodically, and the height of the stems was measured. All the measurements were carried out in triplicate.

3. Results

3.1. The fermented Nile tilapia wastes supernatant

The result in figure (9) shows that the level of pH doesn't change effectively. The same also exists for the lactic acid production and the bacterial growth. These results were due to the scarcity of glucose which is the main source of nutrients for the *Lactobacillus plantarum* bacteria. Consequently, the bacteria were not able to grow up and adapt to use the available nutrients and produce lactic acid.

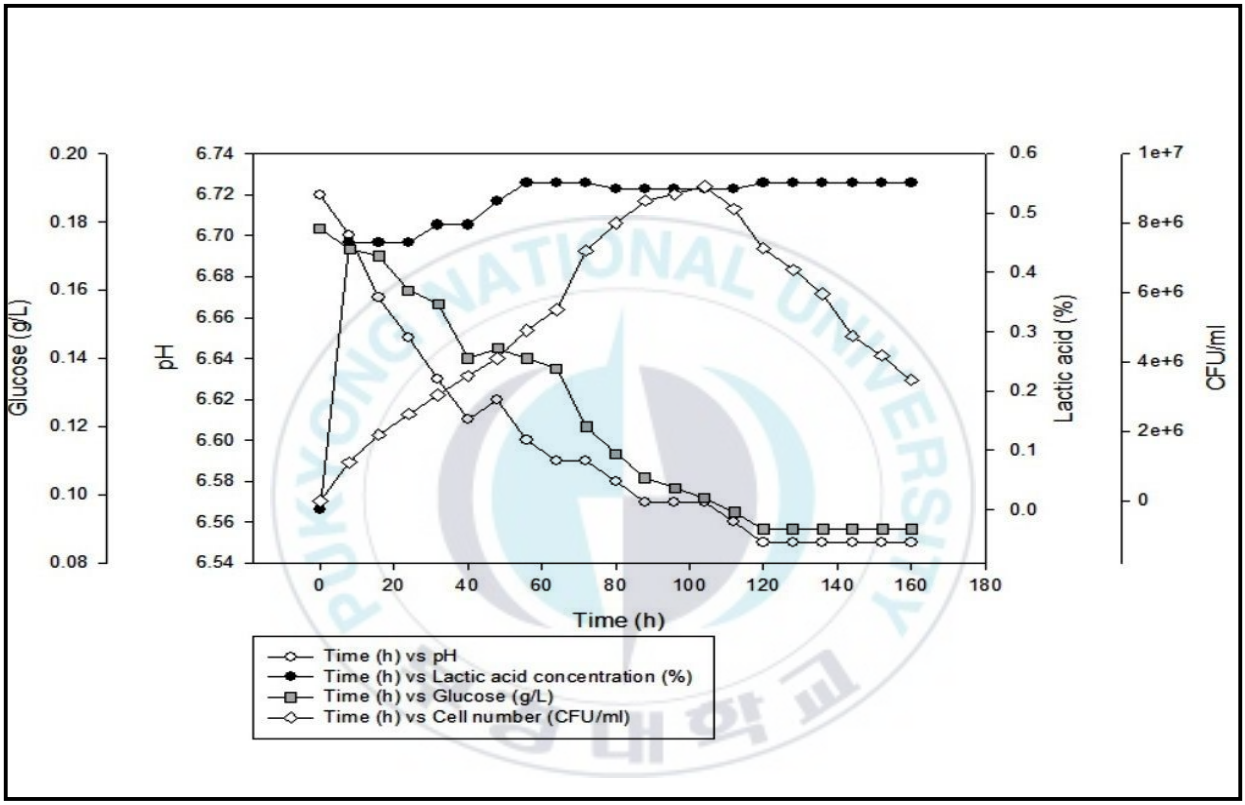

Fig. 9. The analysis of the fermented Nile tilapia wastes supernatant medium

3.2. The fermented Nile tilapia wastes supernatant including 1% glucose

The results in figure (10) show that the decrease in pH level started slowly in the first 50 hours. After approximately 52 hours, the rate of decrease in pH level became fast and it continued decreasing till 240 hours. Finally, it stopped and became stable.

Moreover, the same results illustrate that the production of lactic acid started slowly till the hour 50 when the concentration of the lactic acid was 0.59%. After 100 hours the rate became fast till it reached 1.02% and started to increase by a slower rate till it reached 1.23% after 256 hours.

For the bacterial growth, the results show that it started slowly in the first 110 hours, and reaches the maximum after 150 hours. After that, the number of bacteria started fast to decrease for 40 hours and the rate of decrease became slow. Due to the fact that the bacteria needed some time in the beginning to adapt and produce the lactic acid we have these results. But after adaptation the growth rate increased till reached the maximum. At last, it started to die due to the changing in the levels of pH, the produced lactic acid and the amount of available glucose.

The glucose consuming ratio, as the results show, was a little bit slower since the beginning of the incubation. It had a small two dips after 50 and 100 hours respectively, and that is because of the decreasing in pH level which affect the bacterial activity.

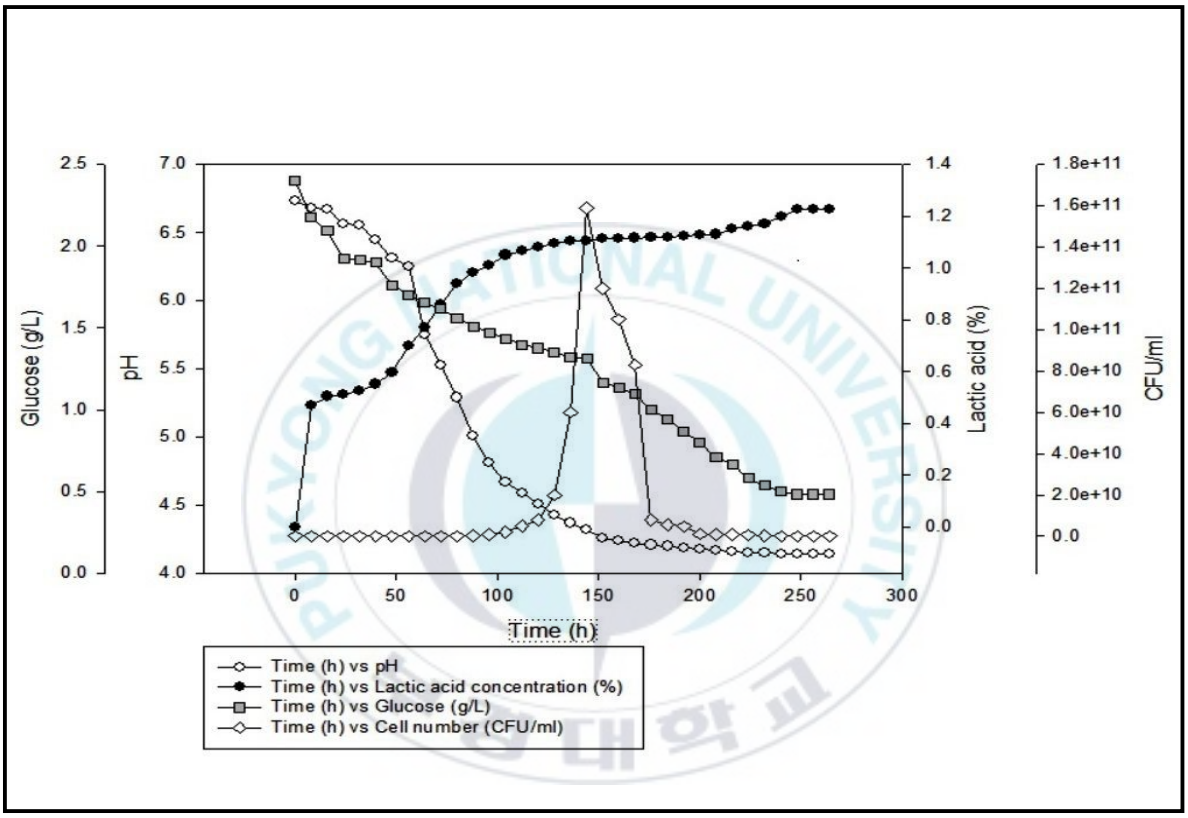

Fig. 10. The analysis of the acid-hydrolyzed Nile tilapia wastes including 1% glucose supernatant medium.

3.3. The fermented Nile tilapia wastes supernatant including 2% glucose

The results in figure (11) indicate that the decreasing in pH level started so fast at the first 48 hours. After that, the rate of decreasing in pH level became slow and continued decreasing till it reached 3.76 after 144 hours of incubation and then stopped decreasing and became stable.

The same results clarified that the production of lactic acid started fast since the start of incubation till it reached 1.61% after 152 hours.

And for the bacterial growth, the results show that it started faster than the two other samples (A and B) and it reached the maximum after approximately 100 hours. Then it started to decrease by a high rate a little bit, and after 130 hours of incubation the decreasing rate became slow. These results prove that the amount of available glucose in the sample was enough for fast adaptation of the *Lactobacillus plantarum* bacteria to grow and produce lactic acid.

The glucose decreasing ratio as the results display was fast since the beginning of the incubation till the first 48 hours. The rate slowed down after that for 24 hours, but it increased again after 75 from the incubation.

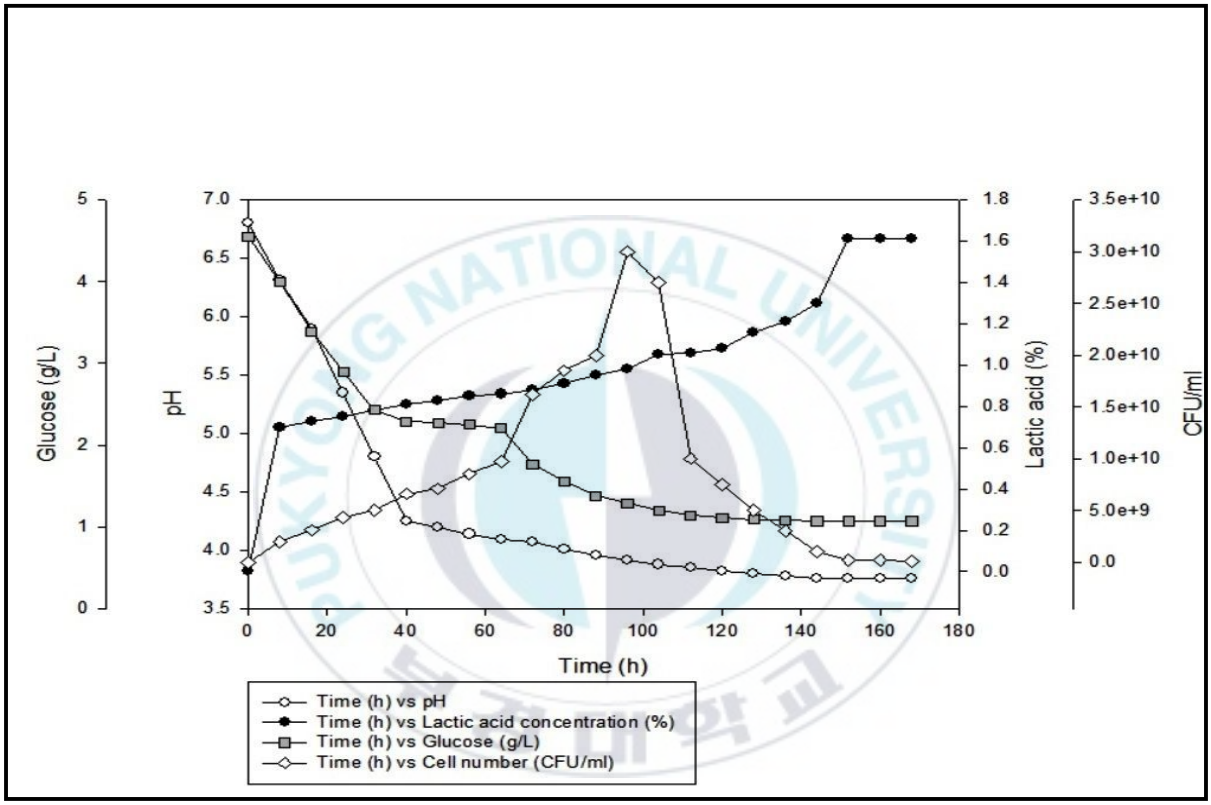

Fig. 11. The analysis of the acid-hydrolyzed Nile tilapia wastes including 2% supernatant medium.

3.4. Comparison with other low-cost medium for different species

In order to evaluate the productivity of the lactic acid from the acid hydrolyzed tilapia waste was compared with the productivity of other species in a low-cost medium.

This comparison depended on the yeast extract (YE) and it is known as the cheapest and common media for bacterial growth. And as it was clarified in the schedule, the glucose concentration in all the other studies was 50-100 g/L but in this study was 0-20 g/L and that explain the huge difference in the productivity between the studies. But if we multiple the amount of glucose in this study with 100 g/L, the results would be the similar. Take for instance, the concentration of 10g/L glucose produced 12.28 g/L lactic acid. If we multiple 10 times the 100 g/L, glucose will produce 122.8 g/L lactic acid. Applying the same method for the concentration of 20 g/L glucose which produced 16.1 g/L lactic acid and if we multiple the amounts in 5 to achieve the 100 g/L glucose it will produce about 80.5 g/L lactic acid.

For the long time of fermentation, the temperature was 30˚C, but for the other studies it was 37-42˚C. Furthermore, the number of species and the activity of the microorganism play an important role in this issue.

Microorganism	Nutrient	Substrate	Time (h)	LA (g/L)	Fermentation temperature	Reference
Lactobacillus plantarum	Acid hydrolyzed Tilapia waste	-	160	5.5	30˚C	This work
		Glucose 10g/L	264	12.28		
		Glucose 20g/L	168	16.1		
Lactobacillus rhamnosus NBRC3863	YE20g/L	Glucose 100g/L	36	87	42˚C	Min-Tian Gao et al. (2006)
Lactobacillus casei ATCC10863	Peptone10g /L +YE5g/L	Glucose 50g/L	30	41	38˚C	Esabi et al. (2003)
Lactobacillus casei NRCCB-441	YE22g/L	Glucose 100g/L	48	100	37˚C	Hujanen and Linko (1996)
Lactobacillus rhamnosus ATCC10863	YE 15g/L	Glucose 150g/L	72	86	N/A	Kwon et al. (2000)
Lactobacillus rhamnosus NRRL-B455	YE10g/L	Glucose 50g/L	40	24.8	N/A	Nancib et al. (2005)

Table 2. Comparison of lactic acid productivity between different species in low-cost media

3.5. Seed germination test results

As the results show, the best results for the seed germination test were for the dilutions at 10‰ and 3‰ respectively, but for the dilution of the 6‰ the results was less than the control sample. Also the results cleared that the diluted solution with 10‰ salinity was better than the 3‰ salinity solution. These results, according to the high concentration of the nutrients in the 10‰ salinity solution than the other concentrations. Also the percentage of the salinity in the 10‰ solution is near to the salinity of the human body solutions which is 9‰.

Test / Dilution	3‰	6‰	10‰
RSG (%)	100	80	100
RRG (%)	75	45	95
GI (%)	75	36	95

Table 3. The results of the GI test for the different dilutions according to the salinity level

3.6. Fertilizing ability

According to the germination test and the results which clarified that the dilution of 10‰ salinity gave the best results against the control sample, so the hydroponic culture system test was held for the 10‰ diluted fermented Nile Tilapia fish waste broth against the control. The results show that the experimental sample was better than the control sample as after 10 days the length average of stems was 10.3 cm for the experimental sample and 9.1 for the control sample. These results were similar and a little bit better than other experimental media which used biodegraded fishmeal waste water and fish wastes for the same plant.

Plant	Experimental media	Duration (days)	Length of stem (cm)	Reference
Barley	Fish waste	10 days	N/A	Kim et al. (2010)
	FMW		9.4	Byeong and Joong (2012)
	Fish waste		9.8	Dao and Kim (2011)
	Nile Tilapia fish waste		10.3	This study

Table 4. Comparison between the effects of different media which used as a fertilizer for barley plant growth in hydroponic system

Discussion

4.1. The acid-hydrolysis

Generally, most of the studies use the enzymatic hydrolysis for the nutrients. However, in this study the acid-hydrolysis of nutrients was used to reduce the cost of lactic acid production. Also using the enzymatic hydrolyses have many disadvantages like most of the enzymes are relatively unstable at high temperatures, the costs of enzyme isolation and purification are high and it is quite difficult to recover them from the reaction mixtures.

4.2. The microorganism

In this study the aerobic bacteria were used because most of the fermentation studies used anaerobic bacteria, so we wanted to be non traditional. Therefore the *Lactobacillus plantarum* bacteria were used because of its importance in food products, silage production, producing antimicrobial substances and it is used as a probiotic.

4.3. The lactic acid production

Three different samples were used in the process of lactic acid production and all of them were the supernatant of the acid-hydrolysed Tilapia waste. The acid-hydrolysis with concentrated H_2SO_4 was used to break the chemical ponds, to extract the nutrients from the Tilapia wastes to make it easier to consume by the bacteria, and the supernatant was used after adjusting the pH.

On the one hand, the acid-hydrolyzed Nile tilapia wastes sample results clarified that it was useless to use it for the production of lactic acid as it produced a little amount of acid due to the absence of sugar which is the main source of feeding for the *Lactobacillus Plantarum* bacteria for growth and fermenting the sugars to produce lactic acid. On the other hand, the acid-hydrolyzed Nile tilapia which included in it glucose 1% and 2% respectively, the production of lactic acid was much better, as it was 12.28 g/L and 16.1g/L respectively. And these results are due to the availability of the glucose. The amount of produced lactic acid is small because there was no enough glucose for the bacteria to consume and produce lactic acid. In another experiment which was held to produce enough amount of fermented Nile tilapia waste supernatant in order to use it in the hydroponic culture test

(results not shown in the study), amount of 50g/L of glucose was used. In this experiment produced amount of lactic acid was 45g/L and this result is better than other studies which were held by another species of bacteria with the same amount of glucose (Esabi et al. 2003 and Nacib et al. 2005).

Moreover, as the results illustrate that the maximum growth of *Lactobacillus plantarum* bacteria was in the fermented Nile tilapia waste included 1% glucose and it was much higher than fermented Nile tilapia waste supernatant sample and the other sample which included 2% glucose. We can consider it good result if we are interested in using the Nile wastes supernatant as an agar medium or just for bacterial growth.

4.4. The effect of the fermentation temperature

For the temperature, indeed it has an effect on the duration of fermentation as it was clarified in the other studies, when they used temperature 37-42˚C; it decreases the duration of fermentation to 30-72 hours, only. Also, during this study there was an experiment (not shown in the results) using the same method in preparing the supernatant from the Nile tilapia waste. It was inoculated with the *Lactobacillus plantarum* bacteria without adding glucose and then incubated at 37˚C. The results of

this experiment shows that after 16 hours only the bacteria produced 5.5 g/L lactic acid.

4.5. The fertilizing ability

As the results of the fertilizing ability experiment which was held in the hydroponic culture system on the Barley plant clarified that the fermented Nile Tilapia fish waste broth can be used as a liquid fertilizer for the plants when the diluted concentration with salinity of 11‰ used. This result according to the fact that the tilapia fish contains high value of proteins and minerals, so consequently the wastes will still contains high percentage of the nutrients because the wastes are about 60% from the whole fish.

Contents and its Percentage			
Nutrient		Vitamins	
Protein	40%	Vitamin B12	26%
Calories	5%	Vitamin B6	20%
Total Fats	3%	Niacin	8%
Cholesterol	19%	Vitamin A	0%
Fiber	0 %	Vitamin C	0%

Table 5. Percentage of the daily value per 100 g. of Nile tilapia fish for the nutrients and vitamins

Minerals and its percentage	
Selenium	78 %
Phosphorus	20%
Potassium	11%
Sodium	2%
Calcium	1%
Iron	4%

Table 6. Percentage of the daily value per 100 g. of Nile tilapia fish for the minerals

Moreover, it would be better if another research try to separate the acid before using the broth as a fertilizer to be suitable in the cases of the plants that can't stand the salinity.

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